Research Article

Bone mesenchymal stem cell-conditioned medium decreases the generation of astrocytes during the process of neural stem cells differentiation

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Objectives: The aim of this study was to investigate the effect of bone mesenchymal stem cell (BMSC) conditioned medium (CM) and Bone morphogenetic protein-4 (BMP-4) on the generation of astrocytes during the process of NSCs differentiation.
Design: Neural stem cells (NSCs) were grown under different culture conditions.
Setting: The First Affiliated Hospital of Anhui Medical University, Hefei, China.
Outcome Measures: The study consisted of four groups: NSCs cultured under control conditions (group 1) or with the addition of BMSC-CM (group 2);(BMP-4) (group 3) or both (group 4).The expression of glial fibrillary acidic protein (GFAP) was detected by immunocytochemical staining and Western blotting.
Results: The expression of GFAP was higher in Group3 and lower in Group 2 compared to that in Group 1. The expression of GFAP in Group 4 was intermediate between that of Group 2 and Group 3.
Conclusions: These results suggest that BMSC-CM can decrease the generation of astrocytes and that the inhibition of the (BMP-4) /Smad1/5/8 signaling pathway may be the underlying mechanism. This phenomenon may be mediated by increasing the expression of Smad6.

Keywords: Neural stem cell differentiation, Astrocytes, BMP-4/SMAD signaling pathway, Bone morphogenetic protein-4, Bone mesenchymal stem cells conditioned medium

Introduction

Spinal cord injury is among the main causes of motor and sensory dysfunction, and there are no effective treatments.[1] Although neural stem cells (NSCs) are present in the adult central nervous system in mammals, injured mammalian spinal cords exhibit a limited capacity to recover spontaneously. Many researchers have investigated methods to improve neural function, and recent work suggests that cell transplantation may be promising approach for treating spinal cord injury.[2] Many cell lines, including neural stem/progenitor cells (NS/PCs), both of which exhibit acceptable levels of differentiation, may be considered therapeutic candidates for spinal cord injury.[3] Both the innate properties of the cells and host-related factors influence the survival and differentiation of transplanted cells. Transplanted NSCs need to differentiate into cell lineages that aid remyelination, axon guidance, and functional synaptic replacement to promote the generation of the spinal cord. In previous research, after NSCs were transplanted into injured adult rat spinal cords, the majority of cells differentiated into astrocytes, with fewer numbers of neurons and oligodendrocytes detected.[4] Studies have also showed that the expression of bone morphogenetic proteins (BMP-2/4/7) was increased in cases of spinal cord injury.[5,6] Some groups demonstrated that the cell fate of transplanted cells was altered from neurons to astrocytes when NSCs in culture were exposed to BMPs.[7] The biological function of BMP may contribute to the undesirable generation of astrocytes after NSCs transplanted into

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injured spinal cord. The aforementioned biological function of BMPs was shown to be mediated by binding to type I serine/threonine kinase receptors, including Ia and Ib, and type II receptors.[8] Studies have also revealed that the BMP Ia receptor signaling pathway up-regulated the expression of glial fibrillary acidic protein (GFAP).[9,10]

Smad proteins are important transcription factors of the BMP signaling pathway. Smad family comprises three different subclasses of proteins.[11] According to previous research, activation of the BMP-Smad1/5/8 signaling pathway increases the generation of astrocytes during NSC differentiation. Research also suggested that the BMP-Smad1/5/8 signaling pathway could be inhibited by inhibitory-Smads (e.g. Smad6).[12]

Bone mesenchymal stem cells not only differentiate into osteocytes, chondrocytes, and adipocytes but also have significant neurotrophic properties.[13,14] Previous studies demonstrated that transplantation of bone mesenchymal stem cells (BMSCs) results in significant functional recovery in cases of central nervous system injury.[15,16] In addition, soluble factors secreted by BMSCs promot the differentiation of neurospheres into neurons and oligodendrocytes.[17] However, the mechanisms underlying such differentiation are not clear. A previous study reported that BMSCs upregulated the expression of inhibitory Smads (e.g. Smad6) and downregulated the expression of receptor-regulated Smad (R-Smad) and Co-Smad.[18]

The aim of present study was to investigate the effect of BMSC conditioned medium (BMSC-CM) on the generation of astrocytes during the process of neural stem cell (NSC) differentiation. We hypothesized that the generation of astrocytes during this process would decrease, due to the inhibition of the BMP signaling pathway, as we have previously found that BMSC-CM up-regulates the expression of inhibitory Smad (I-Smad6) and down-regulates the expression of Smad1/5/8.[19]

Materials and Methods

Reagents and animals

The following reagents were used: Epidermal growth factor and basic fibroblast growth factor (PeproTech, Rocky Hill, NJ, USA), B27 (Invitrogen, Carlsbad, CA, USA), FITC-labeled secondary antibodies to mouse IgG and poly-L-lysine (Sigma, Shanghai, China), DMEM and DMEM/F12 (Gibco BRL, Gaithersburg, MD, USA), Fetal bovine serum (HyClone, Logan, Utah, USA), Anti-GFAP (Sigma, Shanghai, China), Mouse anti-rat flow antibodies (CD29,34,45,90) (eBioscience, CA, USA), Sprague-Dawley (SD) rats (Institute of Experimental Animal Science, Anhui Medical University, Hefei, China).

Culture of NSCs in vitro

NSCs were isolated from the cerebral cortex of 1–2-day-old newborn SD rats. Cells were dissociated and grown as neurospheres in proliferation medium containing DMEM/F12, with 2% B27 supplement, epidermal growth factor (20 ng/ml), and basic fibroblast growth factor (10 ng/ml). Thereafter, the cells were seeded in 75 cm^2 flasks at 10^5 cells/ml. The cells were then incubated in a cell culture incubator at 37° C in humidified atmosphere of 5% CO_2. The medium was changed every 4 days. The cells were maintained in the culture system for 7 days. The cells proliferated and formed many suspended neurospheres. The neurospheres were collected for passage for further analysis when they reached a diameter of approximately 100 μm in diameter. To subculture neurospheres, the cultures were centrifuged gently in a centrifugal tube at 1000 rpm for 5 min. Neurospheres at passage 2 were dissociated into single cells and plated to coverslips for differentiation under different experimental conditions. And these experiments were repeated every 7 days.

Isolation and proliferation of BMSCs

BMSCs were isolated from the rats, according to reported methods.[20] Briefly, rats were sacrificed using pentobarbital sodium, and BMSCs were then collected from femurs and tibias. To lyse the erythrocytes, the suspension of collected tissues was centrifuged at 500×g for 5 min. The deposited cells were re-suspended in a mixture of Krebs buffer and 0.83% NH4Cl for 10 min at 4° C. After performing this procedure a second time, the tissues were washed twice with PBS, and the cultures were centrifuged at 500×g for 5 min. After the precipitated cells were re-suspended with the DMEM containing 20% fetal bovine serum, and the cells were seeded at 10^6 cells/ml in a 75-cm^2 glass flask. BMSCs were cultured in a cell culture incubator at 37° C in humidified atmosphere of 5% CO_2. The medium was replaced after 48 hours and then changed every 4 days. BMSCs were passaged using a mixture of PBS and 0.25% trypsin after reaching at least 90% confluence. The cells were then re-plated in the glass flask, with 10^5 cells/ml. CD34, CD45 (hematopoietic stem cell surface antigen), CD29 and CD90 (mesenchymal stem cell surface antigen) were used to identify BMSC via flow cytometry.[21] Flow cytometry was used for the identification of cell-surface antigens of BMSCs (Fig. 1). BMSC-CM was obtained as previously

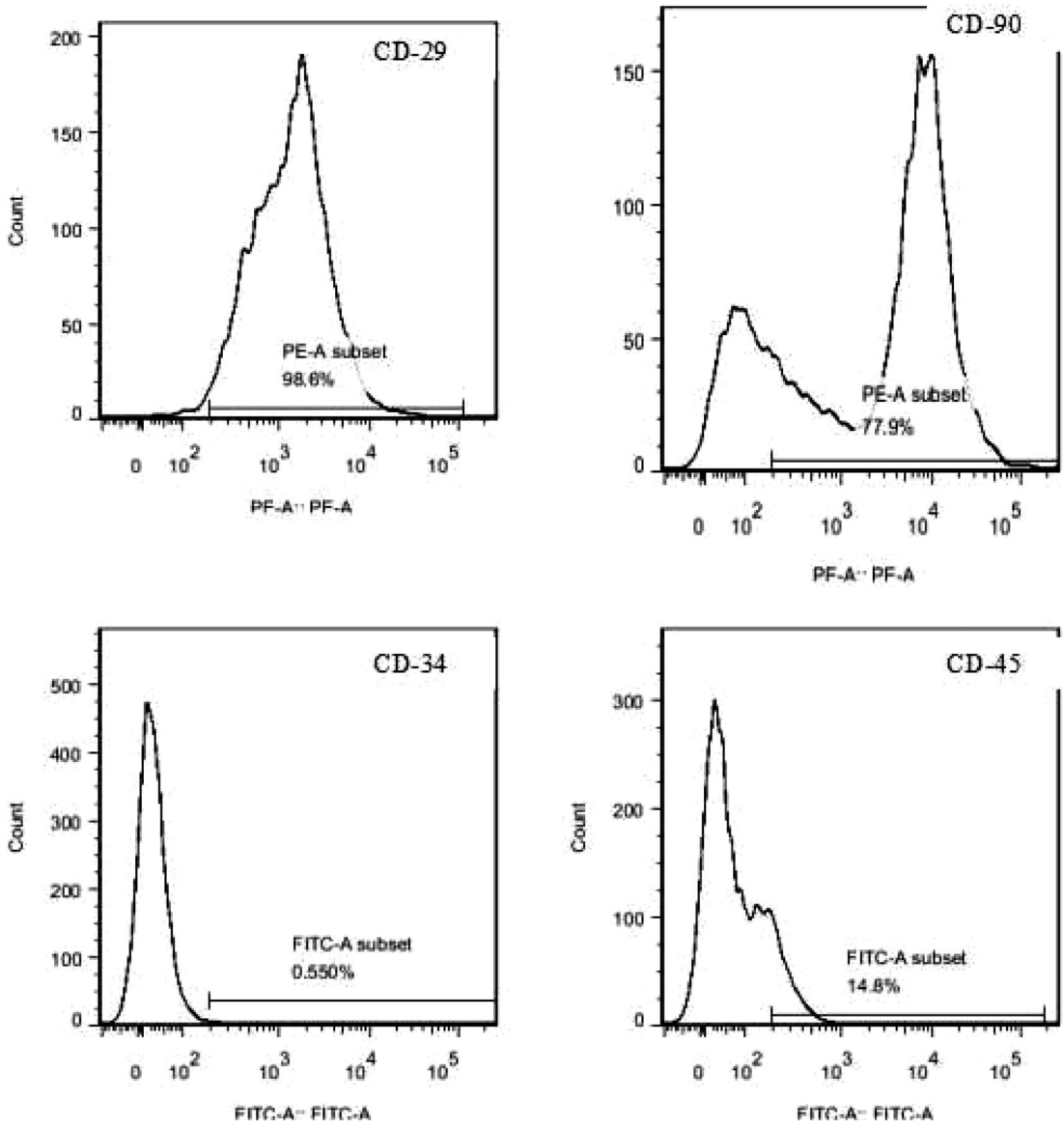

Figure 1. Characterization of BMSCs. Surface antigens of BMSCs were analyzed by flow cytometry.

described.[20] When the BMSCs grew to at least 90% confluence, the medium was removed and washed three times with Krebs buffer. Finally, the BMSCs were cultured with D-medium for 12–24 hours. The BMSC-CM was obtained using a 0.22 μm filter.

Differentiation of NSCs in vitro

Cells at passage 2 were used to investigate the differentiation of NSCs under different conditions. The dissociated NSCs were plated onto poly-L-lysine-coated coverslips in 24 well plates at a density of 3×10^4 cells/coverslips. The proliferation medium was used to promote the adhesion of the neurospheres for 1 hour. The medium was then changed and replaced with different differentiation media according to the experimental design, as follows: (A) Group 1 (differentiation medium consisting of DMEM/F12 and B27+NSCs) (B) Group 2 (differentiation medium consisting of DMEM/F12 and B27+NSCs+BMSC-CM;(C) Group 3 (differentiation medium consisting of DMEM/F12 and B27+NSCs+BMP-4; and (D) Group 4 (differentiation medium consisting of DMEM/F12 and B27+NSCs+BMP-4+BMSC-CM. The medium was changed every 3 days. The cells were allowed to differentiate for 7 days before further experiments.

Immunostaining of cultures

The number of cells expressing GFAP was examined by immunocytochemistry. The cells were fixed in 4% paraformaldehyde for 15–20 min at room temperature and then washed three times with PBS for 5 min. To block nonspecific antigens, the cells were treated with 0.3% Triton-X

100 containing 10% normal goat serum at room temperature for 30 min. The cultures were incubated overnight with anti-GFAP at 4° C. They were then washed three times with PBS for 5 min, followed by incubation with FITC-labeled specific secondary antibodies at 37° C for 40–60 min. The cells were then washed again three times with PBS for 5 min. DAPI was used to stain the nuclei at room temperature for 5–10 min. Finally, the slides were rinsed three times with PBS for 5 min. The samples were analyzed via fluorescence microscopy. The experiments were carried out in triplicate.

Cell count assessment and statistical analysis

A fluorescence microscope was used to examine the immunostained cells. To determine the number of cells that expressed GFAP, 20 fields of each slide were randomly selected by an individual who did not participate in the experiments. The results were expressed as percentages of the numbers of cells positive for GFAP (astrocytes) compared to the total numbers of cells positive for DAPI. Statistical analyses were carried out using one-way ANOVA and SNK method to compare the difference among different groups with SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). The results are presented as the mean ± SD.

Western blotting

Western blotting was performed as follows: Adherent NSC cultures were treated with trypsin-EDTA (0.25%, Invitrogen) and harvested manually from the flask surfaces after differentiation for 7 days. The trypsinized differentiated NSCs were collected by centrifugation, washed with PBS, and lysed in T-PER (Pierce) containing a Halt proteinase-phosphatase inhibitor on ice for 15 min. They were then centrifuged at 15 000 g for 20 min, and the final supernatant was collected for Western blotting. Protein concentrations were determined using a microbicinchoninic acid assay (Thermo Scientific, Rockford, IL, USA.) Then, 15 μg of protein samples from each whole cell extract were separated by 5–20% gradient SDS-PAGE and transferred onto a nitrocellulose membrane. Before incubation with the primary antibody overnight at 4° C, the membranes were placed in 5% Blotto in TBS with 1% Tween 20 for 1 hour at room temperature (see below for the concentration of the antibodies). The membranes were washed in TBS-T, and secondary antibodies were incubated with the membranes and washed again in TBS-T. Finally, the SuperSignal West Femto Maximum Sensitivity Substrate detection system (Pierce) was used to examine the blots. Immunoblots were stripped and reprobed using Restore Western Blot Stripping Buffer (Pierce). The intensity of the target protein was normalized to the intensity of actin.

Results

Identification of Bone Mesenchymal Stem Cells

Flow cytometry experiments shows BMSCs were positive for CD 29 and CD 90, and negative for CD 34 and CD 45 (Fig. 1), which is consistent with the minimal criteria for defining BMSCs.[22]

Induction of NSC differentiation into astrocytes using BMP-4

Astrocytes proliferate in response to central nervous system injury, which results in the formation of glial scars. The expression of BMPs is well known to be upregulated after spinal cord injury. A previous study demonstrated that BMPs could alter the fate of NSCs from neurogenesis to astrocytogenesis.[23] The present study examined whether BMP-4 could alter the fate of cortex-derived NSCs. As shown in Figure 2.1, cells expressing GFAP were more numerous in the Group 3 than in the Group 1. In Group 1, 61.6 ± 6.7% of the total cells were positive for GFAP, compared with 89.7 ± 4.6% in Group 3 (Fig. 2.2).

BMSC-CM prevented NSC differentiation into astrocytes

As described above, to improve functional recovery after transplantation, transplanted NSCs do not differentiate into astrocytes. We hypothesized that inhibiting BMP-4 signaling would prevent the generation of astrocytes during NSC differentiation and that BMSC-CM would inhibit BMP-4 signaling. As shown in Fig. 2.1, the number of astrocytes in Group 2 was significantly decreased compared to that in the other three groups. In Group 2, 43.2 ± 5.8% of cells were positive for GFAP, which was less than that in the control group. In Group 4, 59.8 ± 6.25 of cells were positive for GFAP (Fig. 2.2).

The expression of the GFAP protein in different groups at different points

As shown in Fig. 3, the protein expression of GFAP was assessed by Western blotting analysis in the different groups 3, 6, and 12 days after differentiation (3 DIV, 6 DIV, and 12 DIV, respectively).

Discussion

The purpose of this study was to investigate how BMP-4 and BMSC-CM affect the process of NSCs differentiation.BMP-Smad 1/5/8 signalling has been demonstrated by many groups to promote NSCs differentiation towards GFAP-expressing astrocytes.[24] The

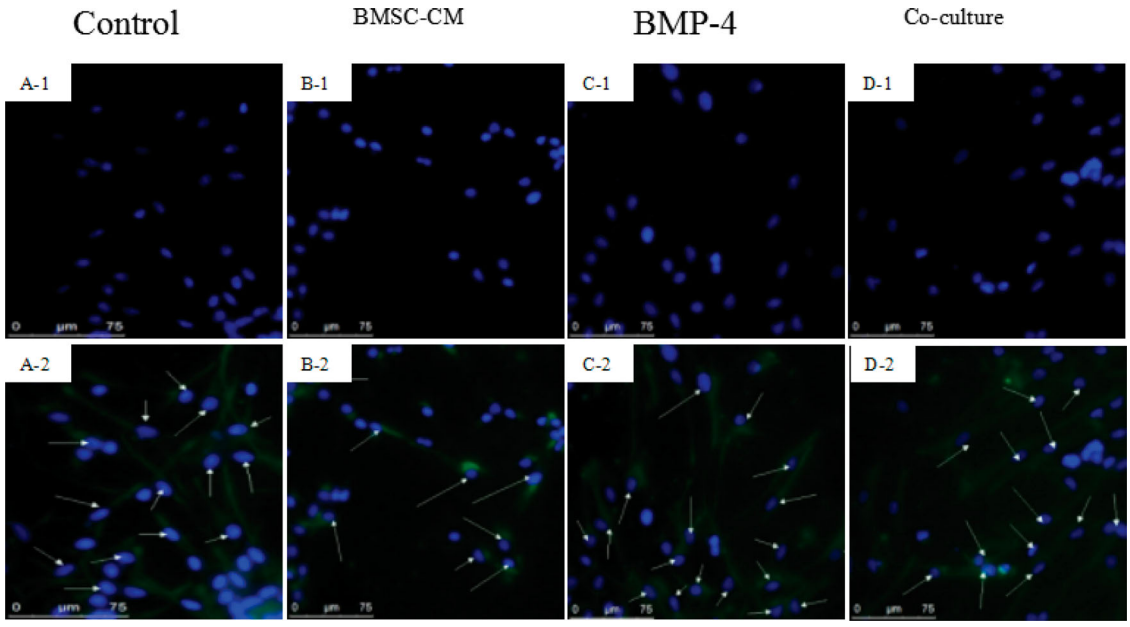

Figure 2.1. Cells expressing GFAP in different differentiation media. In the control group (named Group 1), some cells expressed GFAP (A1 and A2). In contrast, in the presence of BMP-4 (named Group 3), the number of cells expressing GFAP significantly increased (C1 and C2). The number of cells expressing GFAP in the BMSC-CM group (named Group 2) was dramatically decreased as compared to that of the Group 1 (B1 and B2). In the co-culture group (named Group 4), the number of cells expressing GFAP was obviously decreased as compared to that of the BMP-4 group (D1 and D2). Blue (1) = DAPI-labeled nuclei. Green = GFAP-labeled astrocytes. 2 = merges. Bar = 75 μm.

BMP/Smad-dependent pathway is activated by the phosphorylation of receptor-regulated Smad (R-Smad) and inhibited by the dephosphorylation of R-Smad or overexpression of inhibitory Smads (I-Smads).[25] The present study focused on Smad1/5/8 and I-Smad6. And we have found that BMSC-CM could upregulate the expression of inhibitory Smad (I-Smad6) and inhibit the BMP-4/Smad1/5/8 signaling pathway.[19]

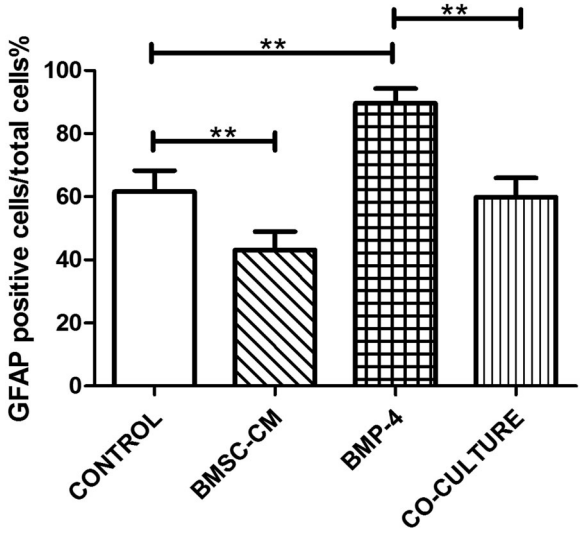

Figure 2.2. Effect of different conditions on NSC differentiation into neurons. The cells were immunostained to detect the expression of GFAP. Then, 20 fields were imaged, and the cells were counted under a fluorescence microscope. The data were analyzed using SPSS software. The data were compared using one-way ANOVA and SNK method. (F=216.71,P < 0.01).**

In the present study, BMP-4 increased the generation of GFAP-expressing astrocytes after NSC differentiation as compared to that in the control group. Rivera *et al.* reported that the generation of astrocytes and oligodendrocytes was not due to selective proliferation of a particular cell type or selective cell death.[26] Lou *et al.* also showed that the BMSC-CM could decrease the expression of GFAP.[17] However, the percentage of GFAP-expressing cells was higher than seen in our study. This difference was probably due to the different concentration of BMSC-released factors in the conditioned medium, which might be caused by the using of different pore size, speed or time of centrifugation during the preparation of the conditioned medium (CM). This hypothesis should be further investigated in subsequent experiments. In the current study, the Western blotting analysis demonstrated that the expression of GFAP increased over time (3 DIV, 6 DIV, and 12 DIV) in the BMP-4 group, whereas this phenomenon was not obvious in the other three groups. Conversely, the results also revealed that BMSC-CM not only decreased the generation of

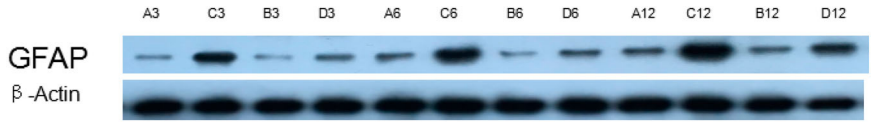

Figure 3. Protein expression of GFAP in each group 3, 6, and 12 days after differ nation. 3 DIV, the expression of GFAP in each group 3 days after differentiation; 6 DIV, the expression of GFAP in each group 6 days after differentiation; 12 DIV, the expression of GFAP in each group 12 days after differentiation. The expression of GFAP in each group: A, Group 1; B, Group 2; C, Group 3; D, Group 4.

GFAP-expressing astrocytes compared to that in the control group but also weakened the effect of BMP-4 on the generation of astrocytes during the process of NSC differentiation. Many groups have shown that the effect of BMP on the generation of astrocytes depends on the activation of the BMP/Smad1/5/8 signaling pathway and that I-Smad inhibited this activation.[27–29]

Taken together, these results demonstrate that BMP-4 enhances the generation of astrocytes during NSC differentiation and that the activation of the BMP-4-Smad1/5/8 signaling pathway may be the underlying mechanism. The BMSC-CM clearly decreased the generation of cells expressing GFAP in the Group 2 as compared to the Group 1. The generation of GFAP-expressing cells was also decreased in the Group 4 as compared to the Group 3. Sun *et al.* also showed that BMSCs increased the expression of the I-Smad.[18] We hypothesize that the increase in the expression of I-Smad6 may be underlying mechanism involved in the phenomenon that the generation of astrocytes was decreased.

In conclusion, the present study showed that BMP-4 increased the generation of GFAP-expressing cells. The BMSC-CM not only decreased the generation of astrocytes but also weakened the effect of BMP-4 on the progression of NSC differentiation possibly through the BMP-4/Smad signaling pathway. However, the mechanism underlying the effect of BMSC-CM on the expression of Smad (both R-Smad and I-Smad) is not clear. Furthermore, the specific components of the BMSC-CM that affect the expression of Smad remain unknown.Additional experiments to investigate these remaining issues are therefore warranted.

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Data archiving

There were no data to deposit.

Conflict of interest

The authors declare no conflict of interest.

Disclosure

The authors declare that they have no competing interests.

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